Do ovarian steroid hormones control the resumption of embryonic growth following the period of diapause in roe deer (Capreolus capreolus)?

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Abstract: Embryonic diapause in the European roe deer includes a period of five months from August to December in which embryonic development is extremely decelerated. Following exit from diapause, the embryo rapidly elongates and subsequently implants. In diapausing carnivores and marsupials, resumption of embryonic growth is regulated by ovarian steroid hormones. In the roe deer, the role of steroid hormones is not known to date. In the present study, progesterone (P4), estradiol-17 (E2) and total estrogens (Etot) were determined in blood plasma and endometrium of roe deer shot in the course of regular huntings between September and December. Steroid hormone concentrations were correlated to the corresponding size of the embryo derived from ex vivo uterine flushing and to the date of sampling. The mean plasma concentrations of P4 (5.4 ± 0.2 ng/ml, mean ± SE, N = 87), E2 (24.3 ± 2.6 pg/ml, N = 86) and Etot (21.7 ± 2.6 pg/ml, N = 78) remained constant over the sampling period and were not correlated to embryonic size. Likewise, endometrial concentrations of P4 (66.1 ± 6.5 ng/ml), E2 (284.0 ± 24.43 pg/ml) and, Etot (440.9 ± 24.43 pg/ml) showed no changes over time. Therefore, it was concluded that ovarian steroid hormones do not play a determining role in resumption of embryonic growth following the period of diapause in the roe deer.

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Original article

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ABSTRACT

Embryonic diapause in the European roe deer includes a period of five months from August to December in which embryonic development is extremely decelerated. Following exit from diapause, the embryo rapidly elongates and subsequently implants. In diapausing carnivores and marsupials, resumption of embryonic growth is regulated by ovarian steroid hormones. In the roe deer, the role of steroid hormones is not known to date. In the present study, progesterone (P4), estradiol-17β (E2) and total estrogens (E_{tot}) were determined in blood plasma and endometrium of roe deer shot in the course of regular huntings between September and December. Steroid hormone concentrations were correlated to the corresponding size of the embryo derived from ex vivo uterine flushing and to the date of sampling. The mean plasma concentrations of P4 (5.4 ± 0.2 ng/ml, mean ± SE, N = 87), E2 (24.3 ± 2.6 pg/ml, N = 86) and E_{tot} (21.7 ± 2.6 pg/ml, N = 78) remained constant over the sampling period and were not correlated to embryonic size. Likewise, endometrial concentrations of P4 (66.1 ± 6.5 ng/ml), E2 (284.0 ± 24.43 pg/ml) and, E_{tot} (440.9 ± 24.43 pg/ml) showed no changes over time. Therefore, it was concluded that ovarian steroid hormones do not play a determining role in resumption of embryonic growth following the period of diapause in the roe deer.

1. Introduction

The european roe deer is a monoestric ruminant species [1]. During the rut in mid-July to mid-August, females (does) ovulate and mating takes place [2,3]. Ovulation results in the formation of one corpus luteum (CL) or several corpora lutea, which secrete progesterone (P4) for the following five months [4], irrespective of the pregnancy status [5]. These five months after the rut season correspond to the time period where the roe deer embryo undergoes an obligate diapause [3]. In mammals, the term diapause is defined as a temporary delay or arrest of developmental of the embryo in the blastocyst stage. During that period the embryo persists in the uterus in a reversible quiescent state that lasts from days to almost a year. The reproductive feature of diapause is widespread and occurs among multiple mammalian taxa [6,7]. Its presence or absence in congenic species [8–10] however leaves its evolutionary development unsettled [11,12]. Interestingly, the roe deer is the only known artiodactyl exhibiting diapause. During the period of diapause in roe deer, embryonic growth is greatly diminished, although mitotic activity is still present at a very low level [13]. To allow fawns to be born in May-June, a normal growth velocity is resumed in December/January, resulting in rapid embryo elongation, subsequent implantation and epitheliochorial placentation [3].

Embryo elongation is a common feature within the order Artiodactyla and occurs prior to implantation. While the development to the blastocyst stage seems to be rather autonomous and is possible under *in vitro* conditions, the development beyond including embryo elongation is not. It is a critical step in development which to date can only be achieved by support of an appropriate maternal environment. In many artiodactyls, embryo elongation coincides with the embryos capacity to prevent the maternal return to cyclicity. Continued luteal progestin production is critical for the maintenance of a growth promoting uterine environment receptive for embryonic signals. The local effect of steroid hormones is hereby transmitted by differential steroid receptor expression of the endometrium [14–16].

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Embryonic signaling and maternal recognition of pregnancy are therefore both decisive for the prolongation of the luteal function in order to interrupt cyclicity and to maintain a progestational endometrial state [17].

The endometrial receptivity for embryo implantation is only given within a short period of time. Historically, this "window of implantation" has been described as the receptive state of the endometrium. In a temporally specific process, the endometrium is primed by exposure to both estradiol-17β (E2) and progesterone of ovarian origin [18]. Adequate remodeling of the uterus also seems to be at least partly dependent on embryo-maternal interactions during the pre-implantation period [19,20]. Hereby, also paracrine actions of the steroid hormones derived from the embryo and/or endometrium as described in pigs are responsible for the modification of the uterine epithelium [21], which finally allow attachment [22].

In Suidae, the elongated embryo secrets estrogens as anti-luteolysin [23–25]. In ruminants, the best-known anti-luteolytic signaling factor of embryonic origin is interferon-τ (IFNτ). Both compounds are discussed to promote embryo proliferation in an auto-/paracrine manner. The roe deer is the only ruminant where neither a luteotropic nor an anti-luteolytic signal, such as IFNτ, is known to date [26]. By displaying a monoestric behaviour, the embryo does not seem to be in need to overcome luteal regression. However, the mechanisms governing embryo growth and developmental velocity in roe deer remain unclear to date.

In diapausing carnivores and marsupials, the growth arrest of the blastocyst is mediated by a non-functional CL and a quiescent uterus [6,27,28]. In both species, a luteotropic signal secreted by the embryo during the time of embryo reactivation is not known to exist. However, it is only after the stimulation of the luteostatic CL by GnRH through the hypothalamic-pituitary-ovarian-axis upon changes in photoperiod or the lack of lactational prolactin inhibition that P4 rises and embryonic development continues. Thereupon, the carnivore and marsupial embryos attach to the endometrium and implant [29–31].

It is well known that luteal P4, the most common gestagen in mammals, elicits the secretory state of the endometrial glands leading to a favourable intrauterine environment [32]. In the pig, conceptus derived estrogens additionally induce the release of secretory vesicles of the endometrial epithelium [33,34]. While a direct effect of P4 on bovine embryo elongation *in vitro* could not be observed, elevated peripheral P4 concentrations induced changes of the endometrium that, in turn, accounted for the advancement of embryo elongation [35]. In particular, steroid hormones changed the quality and quantity of uterine secretions [36,37]. In marsupials, the resumption of luteal function likewise causes endometrial alterations that alter the uterine milieu and determine embryo reactivation [38,39].

As in other diapausing species, the resumption of embryonic growth in the roe deer is associated with increased glandular secretions [40,41]. Various studies have shown that plasma P4 remained elevated throughout the period of diapause, where the embryo is in the blastocyst stage and does not elongate [42,43]. Hoffman et al., 1978 [5] collected monthly plasma samples from 8 pregnant and 3 non-pregnant captive does over the course of one year. According to their observation, plasma P4 levels in pregnant does reached a first peak in August and a second elevation between December and June as compared to non-pregnant does. Total estrogen (E_{tot}) in pregnant animals was lower during August - December compared to January – June. Due to the study design, the corresponding developmental stages and sizes of the embryos were unknown and could not be taken into account to discriminate the period around elongation and implantation. A rise in plasma progesterone levels was likewise detected by Sempéré (1977) [43] in captured does in the presumed period of embryo elongation and placentation (January to February, n = 21 samples) compared to the presumed period of diapause (October to December, n = 25 samples). Unfortunately again, plasma samples were not attributed to embryonic size. In contrast, Lambert et al. (2001) [44] analysed plasma P4 concentrations of hunted does where the corresponding embryos were collected by uterine flushing. The plasma P4 levels of the does with blastocysts (n = 15), expanded blastocysts (n = 3), elongated embryos (n = 2) and implanted embryos (n = 8) did not differ between developmental stages [44].

Aitken (1974, 1981) [45,46] associated embryo elongation with a significant increase in plasma estrogen. This finding was supported by Lambert et al. (2001) [44], who reported consistently low estradiol concentrations throughout diapause and expansion, but increased levels at elongation, which remained high at implantation. Although the administration of estrogen to pregnant roe deer during diapause led to an increased blastocyst diameter compared to control animals, it did not lead to elongation [46].

Taken together from the results published so far, the contribution of steroid hormones on embryonic growth, reactivation and elongation in roe deer are inconclusive. Therefore, the aim of the present study was to investigate the role of P4, E2 and E_{tot} during the period of diapause and reactivation both on the systemic and local endometrial tissue level.

2. Materials and methods

2.1. Sample collection

Samples were collected during regular, authorized huntings in north-eastern Switzerland and southern Bavaria between 09/2016-01/2017 and 09/2017-01/2018. The majority of huntings was scheduled in November, when weather conditions and visibility of the prey are most favourable for the hunters. In December and January, huntings are far less frequent, accounting for the unequal sampling distribution (n = 5 samples in September, n = 10 samples in October, n = 55 samples in November, n = 30 samples in December, n = 2 samples in January). Samples were collected from sexually mature females above the age of 14 months which exhibited corpora lutea. From a total of 102 female roe deer, we were able to collect blood samples from 87 and endometrial tissue samples from 33 does in the studied period covering diapause and reactivation. After a hunt had ended, the shot does were brought to the gathering place for evisceration by the hunters, and the reproductive tracts of female roe deer were removed. The body condition of the does was assessed by visual inspection and recorded. Blood was directly retrieved from the heart by cutting through the cranial part of the sternum. The blood was collected in sterile tubes containing EDTA (Monovette, Sarstedt, Germany), and kept on ice until centrifugation at 2000 *g* at 4 °C for 20 minutes. The plasma was stored at −20 °C until further analysis. In some cases, depending on the location where the bullets had penetrated and the way the animals were eviscerated by the hunters, it was not possible to obtain good quality blood because of stomach content and/or faeces contamination. These blood samples were excluded for the hormone analysis.

Further sample preparation was performed on site in a mobile laboratory van. The reproductive tract was dissected from the mesometrium and the oviducts together with the ovaries were cut off. For the determination of the number of CL and follicles, the ovaries were cut open lengthwise. The number and size of the follicles on the ovarian surface (> 0.8 mm) which were macroscopically visible without magnification was evaluated under a white light. For embryo collection, the uterus was flushed with 2.5 ml of phosphate buffered saline solution (PBS: pH = 7.4; NaCl, $Na_2HPO_42H_2O$, KH_2PO_4, KCl from Merck KGaA and water from Milli-Q® Integral Water Purification System for Ultrapure Water, Merck KGaA). The uterine flushing was immediately evaluated under a stereo microscope (SteREO Discovery Microscope V8, 1:8 Zoom rate, Zeiss) and a photograph of each embryo was taken (Camera Olympus SC50). For embryos in the blastocyst stage, the maximal diameter was recorded, while for elongated embryos the maximal length was measured.

After uterine flushing, the uterus was opened longitudinally and endometrial tissue of the intercaruncular area was carefully collected

(approximately 400 mg per animal). Tissue samples were snap frozen in liquid nitrogen and stored at −20 °C until further analysis.

2.2. Determination of steroid hormone concentrations in plasma samples

The hormones P4, E2 and E_{tot} (as simultaneously determined through an antibody cross-reacting to estradiol-17β, estradiol-17α and estrone by 100%, 70%, and 100%, respectively) were determined in plasma as described earlier by Prakash et al. [47] and Meyer et al. [48]. A competitive enzyme-linked immunosorbent assay (ELISA) was used for the analysis of P4 (antibody as kind courtesy by Franz Weber, LMU Munich, Oberschleissheim, Germany, and 4-pregnen-3,20-dione-3-*O*-carboxymethyloxime horseradish peroxidase as tracer), E2 (antibody E2/2 Pool 1 and 17 beta-estradiol-*6*-carboxymethyloxime horseradish peroxidase as tracer) and E_{tot} (antibody E2/3 POOL 1 and estradiol-17-hemisuccinate horseradish peroxidase as tracer). The antibodies were kindly provided by Physiology Weihenstephan, Technische Universität München, Germany.

In brief, the steroid hormones were extracted from the plasma with 5 ml tert. butylmethylether/ petrol ether (30/70) (AppliChem, Panraec, ITW Companies), which was added to 500 µl plasma in an extraction glass prior to analysis. The ELISA was performed in a 96-well microtiter plate reader (Cytation 3 cell imaging multi-mode reader, BioTek). The tracer and antibody dilution, limit of quantification, inter-assay and, intra-assay variation of the different assays are given in Table 1.

2.3. Determination of steroid concentrations in endometrial tissue samples

For the analysis of steroid hormone tissue concentrations, approximately 100 mg of intercaruncular endometrium was placed into a plastic tube containing approximately 1000 mg of ceramic beads (MagNa Lyser Green Beads, Roche) and 500 µl of 0.9% sodium chloride (NaCl; Merck KGaAl) solution. Subsequently, the mixture was homogenized in a MagNa Lyser (Roche) by shaking for 1 min at 7000. Then, each tube was incubated at 4 °C for at least 1 h. The content of each tube was transferred into an extraction glass. P4 and E_{tot} from the tissue mixture were determined following the same protocol as described for extracted plasma.

The E2 ELISA performed poor specifically regarding accuracy. Therefore, endometrial E2 was quantified by radioimmunoassay (RIA). Exemplary plasma samples were measures with RIA and ELISA and showed a good correlation (R = 0.86), while the ELISA overestimated the quantity by 10-fold. However, the absolute concentrations were comparable to those reported earlier, which were determined by the same ELISA [5].

For RIA, the dried extracts were re-dissolved in PBS 0.1% bovine serum albumin and subjected to radioimmunological determination performed by a sequential assay [49] as previously described [50,51]. The antiserum used was directed against E2-6-carboximethyloxim (CMO)–BSA exhibiting a cross-reactivity of 1.3% for estrone and < 0.01% for the non-phenolic steroids tested. Intra- and inter-assay CV were 7.1 and 17.6%, respectively. The minimum detectable concentration was 25 pg/g tissue.

Table 2
Number of corpora lutea and percentage of the embryos recovered.

n° of corpora lutea per doe	n° of does in relation to the respective n° of recovered embryos			
	0 embryos	1 embryo	2 embryos	3 embryos
1 (n = 4)	1 (25 %)	3 (75 %)	-	-
2 (n = 87)	17 (20 %)	20 (23 %)	50 (57 %)	-
3 (n = 8)	3 (37.5%)	-	3 (37.5 %)	2 (25%)

2.4. Statistics

The log-transformed data of the hormone concentrations were used for statistical analyses using SAS 9.4 (SAS Institute, Inc.,Cary). The data were subjected to least-square analysis of variance using the Mixed Models procedure including the day of sampling as random factor to determine effects of the day of the year and size of the embryo, respectively. The results from SAS are presented as mean ± SE. Graphs were plotted using GraphPad Prism 7.03 (GraphPad Software). A loess regression was performed in RStudio (Version 1.1.456) with ggplot2. The loess regression type allows local fitting with the weighted least squares method. A confidence interval of 95% is indicated by a gray band.

3. Results

3.1. Corpora lutea and embryos recovered

Cyclic does presented between one and three CL. The majority of does (87.9%) had two CL, while only 4.0% and 8.1% presented one and three CL, respectively. The number of embryos recovered from each doe was sometimes smaller than the number of CL. We obtained zero to three embryos from the uterine flushings per doe. Table 2 shows the number of CL and the respective number of collected embryos. The embryos recovered displayed different sizes (Fig. 1a) and developmental stages, ranging from round shaped blastocysts (Supplementary Fig. 1) to different stages of elongation (Supplementary Fig. 2). The diameter of the blastocysts ranged from 0.15 to 4.28 mm (1.2 ± 0.73 mm on average). The length of the elongated embryos ranged from 5 mm to several centimetres (25.1 ± 23.0 mm on average).

3.2. Plasma and endometrial progesterone

The average plasma P4 concentration (n = 87) was 5.4 ± 0.2 ng/ml and ranged between 1.9 and 12.1 ng/ml showing a high inter-individual variance. In the endometrial tissue, concentrations of P4 (n = 21) were around ten times higher than in plasma (Mean = 66,1 ng/g, ranging from 21.5 to 138.8 ng/g). There was no relationship between either plasma or endometrial progesterone concentrations and embryonic size (Fig. 1a, b, c), day of sampling (Fig. 1a, b, c), number of CL (Fig. 2a), number of embryos recovered (Fig. 3a) or number of follicles (Fig. 4a, b).

Table 1
Enzyme and antibody dilution, limit of quantification, quality control and intra-assay variation of the different steroid hormones measured by ELISA.

Steroid Hormone	Tracer dilution	Antibody dilution	Limit of quantification	Inter-assay variation	Intra-assay variation
Progesterone	1:11 000	1:210 000	0.09 ng/ml	11 %	7 %
Estradiol-17β	1:30 000	1:350 000	15.9 pg/ml	13 %	13 %
Total estrogens	1:19 000	1:400 000	7.7 pg/ml	14 %	11 %

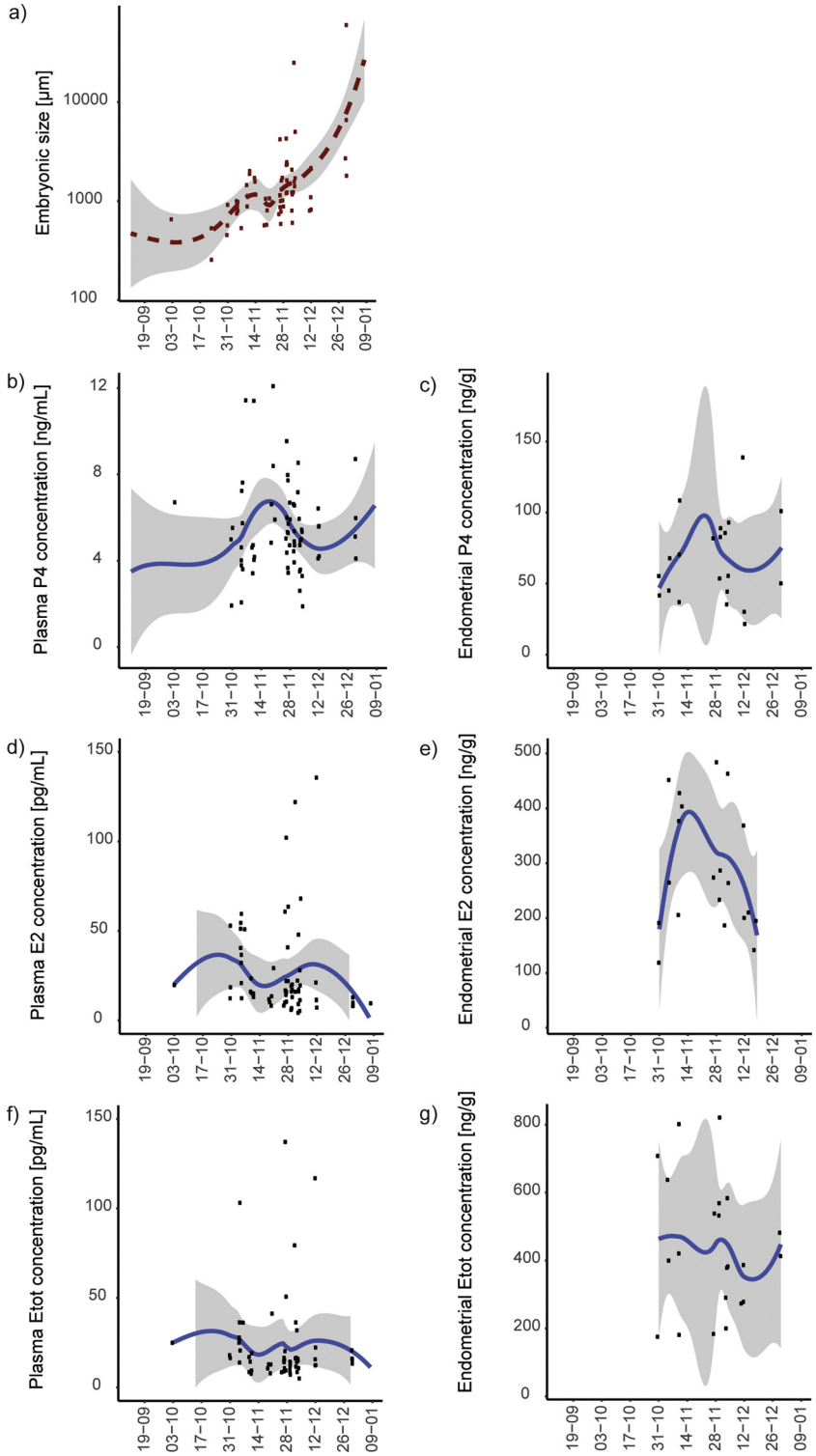

Fig. 1. Relation between a) date of sampling and size of the embryo and relation between date of sampling and corresponding steroid hormone concentrations: b) plasma P4, c) endometrial P4, d) plasma E2, e) endometrial E2, f) plasma E_{tot}, g) endometrial E_{tot}.

3.3. Plasma and endometrial estradiol-17β

Plasma E2 concentrations (n = 86) showed a vast variability between animals and ranged from 4.3 pg/ml to 135.6 pg/ml with a mean of 24.3 ± 2.6 pg/ml. The variability was also evident between samples collected at the same day (Fig. 1d, e) and between samples corresponding to similar embryonic sizes (Fig. 1c, d). Intercaruncular endometrial E2 (n = 21) was around ten-fold higher than plasma E2, ranging from 118.6 to 483.7 pg/g. There was no correlation between the concentration of plasma or endometrial E2 and the date of sampling (Fig. 1d, e), embryo size (Fig. 1a, d, e), number of CL (Fig. 2b), number of recovered embryos (Fig. 3b) or number of follicles (Fig. 4c, d).

3.4. Plasma and endometrial total estrogens

The E_{tot} concentrations (n = 78) presented a high variability

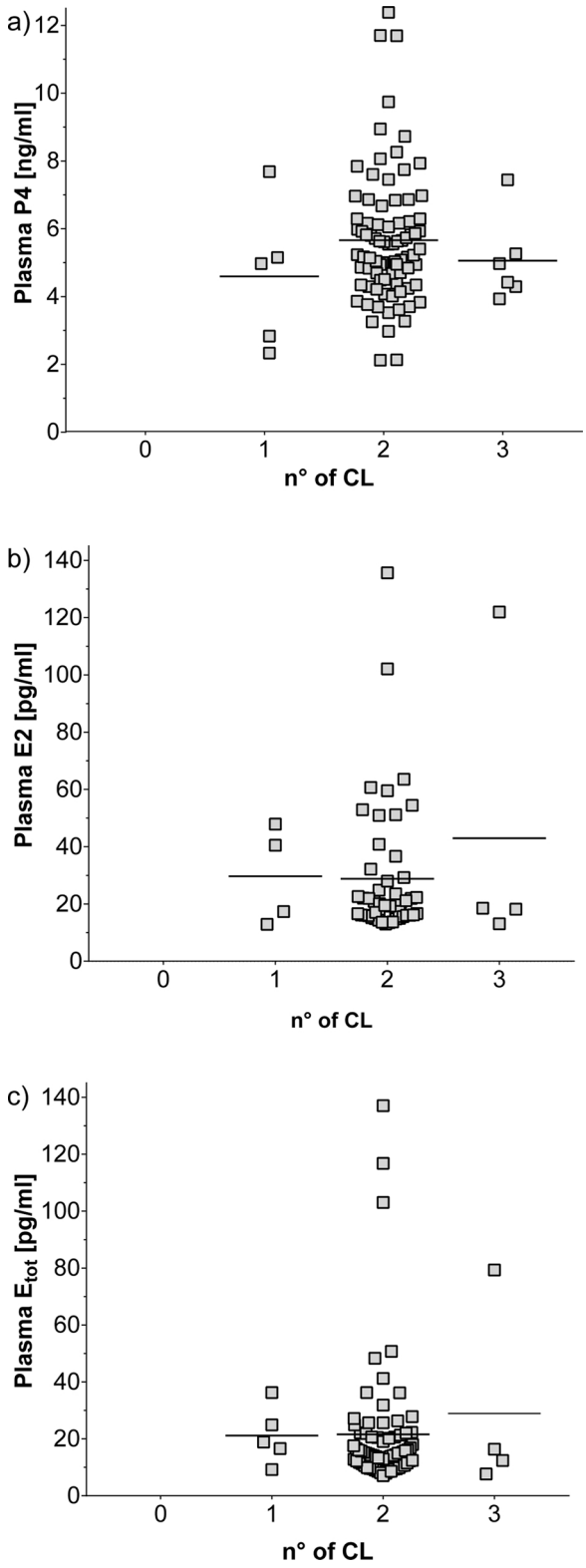

Fig. 2. Plasma concentration of a) P4, b) E2 and, c) E_{tot} related to the number of CL. Mean values are represented by lines. There was no significant effect of the number of CL on any plasma steroid hormone ($p > 0.05$).

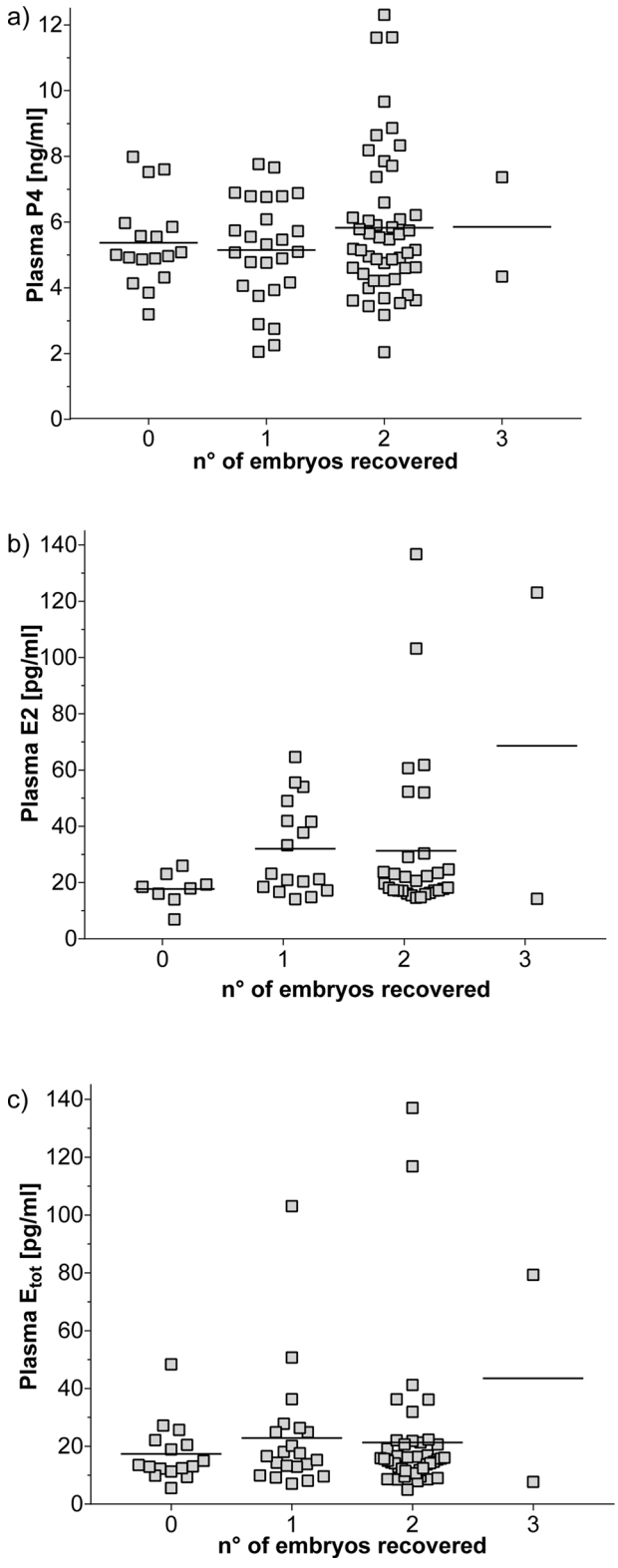

Fig. 3. Plasma concentration of a) P4, b) E2 and, c) E_{tot} and the number of embryos recovered. Mean values are represented by lines. There was no significant effect of the number of embryos on plasma steroid hormone ($p > 0.05$).

ranging from 5.0 pg/ ml to 137.1 pg/ml in plasma and a mean value of 21.7 ± 2.6 pg/ml. Numerically, concentrations of E_{tot} in the endometrium (n = 22) were around 200 times higher than in plasma with a mean value of 440.9 ± 39.29 pg/g. The concentration of plasma and endometrial E_{tot} was neither affected by date of sampling (Fig. 1 f, g), nor by size of the embryo (compare Fig. 1a), nor by the number of CL (Fig. 2c), nor by the number of recovered embryos (Fig. 3c), nor by the number of follicles (Fig. 4e, f).

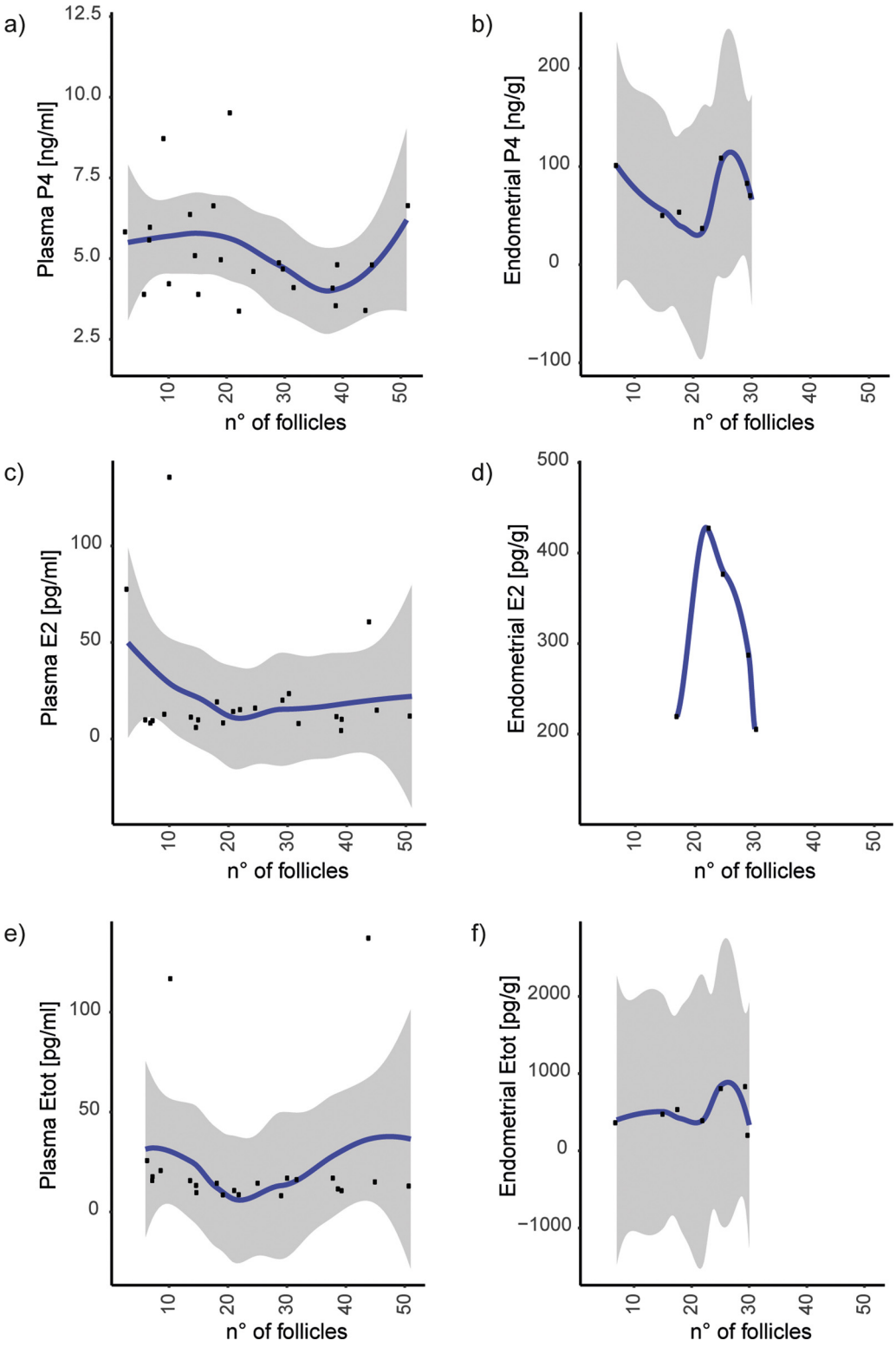

Fig. 4. Steroid hormone concentration in plasma (a, c, e) and endometrial tissue (b, d, and f) related to the number of follicles on both ovaries. There was no significant effect of the number of follicles on neither plasma nor endometrial steroid hormone concentration ($p > 0.05$).

4. Number of follicles

The mean number of follicles per doe was 23.5 follicles (of n = 32 does), where the lowest observed number was 3 and the maximum 51 follicles per ovary. The number of follicles decreased slightly over the sampling period (Fig. 5), corresponding to increased embryonic size (compare Fig. 1a). Nevertheless, no significant differences were found.

5. Discussion

We did not detect any difference in the concentrations of neither plasma nor endometrial P4, E2 and E_{tot} related to embryonic size. We therefore conclude that the constant concentration of P4 during diapause provides a milieu for the blastocyst that enables slow but continuous growth. Thereafter, the resumption of normal growth velocity,

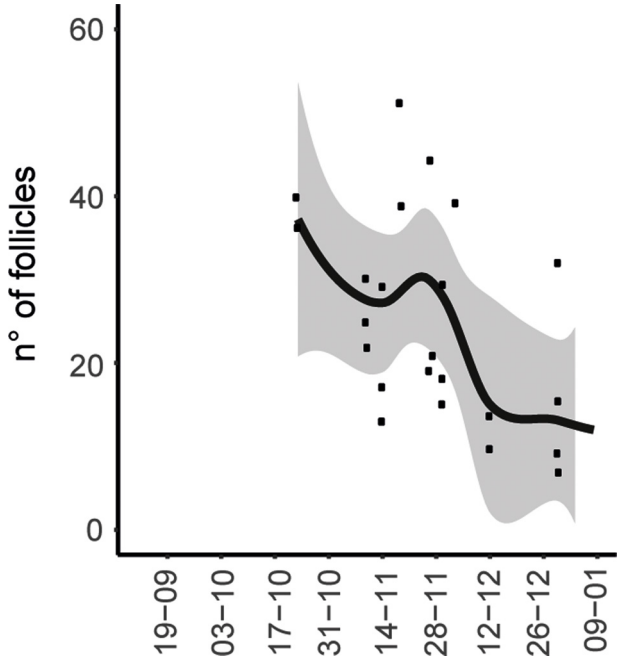

Fig. 5. Number of follicles over the sampling period corresponding to the time of diapause and elongation. There was no significant effect of neither the date of sampling nor the developmental stage on the number of follicles ($p > 0.05$).

that is associated with a pronounced increase in embryo size, finally resulting in embryo elongation, is independent from a lasting change in either P4, E2 or E_{tot}. Maternal support of escape from diapause in roe deer, if necessary at all, is thus not directly driven by ovarian P4.

Likewise, we did not observe the sampling date affecting P4 concentrations, which renders a photoperiod driven P4 stimulation of embryonic growth velocity via the hypothalamic-pituitary-ovarian-axis unlikely. Our findings are therefore in contrast to the control of embryo activation after diapause in mustelids and marsupials, which is regulated by photoperiod-induced changes of luteal function [29–31]. In the tammar wallaby [52,53], the mink [54] and the badger [55,56] the period of diapause was shortened due to a change in photoperiod, concomitant with a highly synchronized birth. Here, the diapausing embryo is maintained in a milieu obviously not suitable for further development. P4 acting on the endometrium is then able to term embryonic growth arrest [57–62]. The assumption that photoperiod does not drive embryonic growth velocity in roe deer is further supported by the great variability of developmental stages encountered around the presumed period of growth resumption at the time of winter solstice on December 21 st. Our observations are in line with the preliminary findings of Lincoln and Guinness (1972) [63]. The latter tested in an experimental approach if the exposure to an artificial light regime that mimicked shorter day length would reduce the period of diapause in roe deer and result in birth prior to the physiological fawning season. For that purpose, two does with observed mating were enclosed in a lightproof shed at defined times each day, thereby progressively reducing the exposure to natural light from beginning to the end of October. Thereafter, increasing daylight length was generated by exposure to artificial light until beginning of December. Of the two does, only one proved to be pregnant and gave birth during the natural fawning season mid of May, indicating that the period of reduced growth had not shortened in response to the artificially advanced winter solstice [63].

In our study, the number of CL explained neither plasma nor endometrial P4 concentrations. Thus, there may be a threshold P4 concentration, being around > 1 ng/ml in plasma, for maintaining pregnancy.

We observed a high individual variation in plasma and endometrial estrogen concentrations being most pronounced in samples corresponding to smaller embryos. A local temporary rise of endometrial estrogens might thus still underlay the present data. Estrogens synthesized by luteal cells during the luteal phase [64] could accumulate due to increased endometrial receptor expression and act locally in the endometrium without being elevated in circulating plasma. However, the number of CL explained neither plasma E2 nor E_{tot}. An endometrial increase in estrogens might also be attributed to hormone synthesis or metabolism by the endometrium itself [34,65–68]

The granulose cells of the follicles are the main source of estrogens and are related to follicular wave patterns. In our study the number of follicles did not explain plasma estrogens. The high variability in follicle numbers and plasma E2 concentrations is in accordance with observations in other species [69,70]. In general, follicular dynamics are not well investigated in other ruminants than in cattle, which exhibit ovarian cycles throughout the year. During anestrus, the ovary exhibits periodic follicular waves without emergence of an ovulatory follicle. Limited data from seasonal breeders such as wapiti (Cervus elaphus), sheep (Ovis aries) and camel (Camelus dromedaries) show that ovarian follicular waves with emergence of a dominant follicle undergoing regression occur during the non-breeding season [71–73]. In contrast, the horse does not display follicular waves in the non-breeding season [74]. For the roe deer it is not known whether follicular waves occur during the long anestrus period, and if so, how long follicular development takes. From our data, we cannot conclude if the observed follicles stem from the same follicular wave or from different waves, nor can we determine the growth phase of the potential follicular wave. Most follicles were present during the presence of a blastocyst stage embryo, mainly corresponding to the sampling period between November and December. During this time, the ovaries exhibited more follicles than later in the season. Follicular development to a certain stage seems to be independent from a central, seasonal photoperiodic or nutritional clue and can therefore be generally observed throughout the season [75]. On the other hand, short days might inhibit follicular development to some extent, which would explain the lower follicle number we observed later in the season in roe deer. Further in depth studies are needed to clarify the role of the resting follicles outside of the season. Since all does in our study exhibited a very good body condition throughout the sampling period, these interindividual differences are more likely to be attributed to either individual differences in hormone synthesis or different stages of follicular development at the day of sampling than to maternal nutritional status.

We observed 10- to 20-fold higher steroid concentrations in the reproductive tissues than in the plasma. Similar observations have been reported in cattle [76] and sheep [77] and can probably be attributed to different matrix properties. In addition to a the local presence of steroid hormone receptors, the direct blood supply by the ovarian vein and the uterine artery could greatly increase the concentration of circulating ovarian steroid hormones in the reproductive tract [78,79].

6. Conclusions

In summary, the results obtained from the endometrial as well as from the plasma analyses do not show any indication that changes in ovarian steroid hormones trigger embryonic reactivation and thus the termination of diapause in the roe deer. Further investigations are necessary to explore the factors controlling the end of the diapause and their sources.

Declaration of interest

All authors confirm that there is no conflict of interest interfering with the impartiality of the scientific work.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.repbio.2019.04.003.

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